

Inflammatory and cytotoxic responses in mouse lungs exposed to purified toxins from building  
isolated *Penicillium brevicompactum* Dierckx and *P. chrysogenum* Thom.

by

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## Abstract

*In vitro* and *in vivo* studies have shown that building-associated *Penicillium* spores and spore extracts can induce significant inflammatory responses in lung cells and animal models of lung disease. However, because spores and spore extracts comprise mixtures of bioactive constituents often including toxins, it is impossible to resolve which constituent mediates inflammatory responses. This study examined dose-response (0.5 nM, 2.5 nM, 5.0 nM, 12.5 nM/g body weight (BW) animal) and time-course (3, 6, 24 and 48hr post instillation (PI)) relationships associated with inflammatory and cytotoxic responses in mouse lungs intratracheally instilled with pure brevianamide A, mycophenolic acid and roquefortine C. High doses (5.0 nM and/or 12.5 nM/g BW animal) of brevianamide A and mycophenolic acid, the dominant metabolites of *P. brevicompactum* and roquefortine C, the dominant metabolite of *P. chrysogenum*, induced significant inflammatory responses within 6 h PI manifest as differentially elevated macrophage, neutrophil, MIP-2, TNF and IL-6 concentrations in the bronchioalveolar lavage fluid (BALF) of intratracheally exposed mice. Macrophage and neutrophil numbers were maximal at 24 h PI; responses of the other inflammatory markers were maximal at 6 h PI. Except for macrophage numbers in mycophenolic acid treatment animals, cells exhibited significant dose dependent-like responses; for the chemo-/cytokine markers, dose dependency was lacking except for MIP-2 concentration in brevianamide A treatment animals. It was also found that brevianamide A induced cytotoxicity manifest as significantly increased LDH concentration in mouse BALF, at concentrations of 12.5 nM/g BW animal and at 6 and 24 hr PI. Albumin concentrations, measured as a non-specific marker of vascular leakage, were significantly elevated in the BALF of mice treated with 12.5 nM/g BW brevianamide A/animal from 6 to 24 hr PI and in  $\geq 5.0$  nM/g mycophenolic acid-treated animals at 6 to 24 hr PI. These results suggest

that these three toxins from *Penicillium* species common on damp materials in residential housing provoke compound-specific toxic responses with different toxicokinetics. Moreover, that these toxins can stimulate significant inflammatory responses *in vivo* might help explain some of the indoor effects associated with *Penicillium* spore exposures in indoor environments.

## Introduction

It is generally accepted that there are population health effects associated with mold exposures in outdoor air. Some 10% of Americans are allergic to *Cladsoporium* spp. spores while some 8% of emergency admissions to hospitals for asthma onset are related to outdoor spore exposures (NAS 2000). Health risks associated with mold exposure in water damaged buildings are also well established. In a recent report, a US National Academy of Sciences committee (NAS 2004) concluded that there is sufficient evidence of an association between exposure to molds in damp indoor environments and upper and lower respiratory tract symptoms, a position more recently echoed by Horner (2005). Amongst those symptoms linked to mold growth in damp buildings are rhinitis, eye irritation, asthma and cough.

An unexplained difference between outdoor and indoor mold exposure outcome is that the symptoms associated with indoor exposures that cannot be attributed to allergy alone (NAS 2004). This was observed in the earliest epidemiological studies (Dales et al. 1991a, b). It has been speculated that this is because the mold species which grow and sporulate on wet building materials comprise a narrow range of toxin-producing species. These epidemiological signals from moisture damaged buildings are linked to exposures to materials from a consortium of toxin-producing species that also contain antigens and beta 1, 3 D glucan. These are mainly anamorphic Ascomycetes, including species of *Aspergillus* (especially *A. versicolor*) and *Penicillium* subgenus *Penicillium* (especially *P. aurantiogriseum*, *P. brevicompactum*, *P. chrysogenum*) and other species including *Stachybotrys chartarum*. Which species dominate depends on the wetted material, environmental conditions that prevailed while there was sufficient moisture for growth, and, to some extent, climate (Flannigan & Miller 2001).

When there was actual mold growth in a large study of Canadian houses, exposure to fungal materials in Canadian houses comprised spores (~30% of airborne fungal glucan from concurrent samples), spore and hyphal fragments (~30%) and fragments smaller than can be recognized by microscopy (~40%; calculated from Foto et al. 2004 and 2005; Horner, 2005). These materials contain species- and strain- dependent mixtures of toxins, glucan and proteins, which can penetrate into proximal lung airways. This apparently results in inflammatory and allergic responses in lung cells. However, clarification of the mechanisms associated with respiratory disease due to indoor fungal exposures is still in its infancy (Jussila et al. 2002). There are data on the rodent lung responses to pure 1, 3  $\beta$  glucan indicating that the fungal glucans in indoor air (Foto et al. 2005) are the most potent form (Young et al. 2003). There are few studies in experimental animals of the effects of low molecular weight compounds from fungi that grow on building materials. To date, only the metabolites of *S. chartarum* have been studied. The macrocyclic trichothecenes produced by some strains of this species result in a range of effects on both type II and alveolar macrophage cells (Miller et al. 2003). Recent studies have found the non-cytotoxic compounds from the other chemotype of this species to be potentially inflammatory (Rand et al. 2005).

As noted above, the dominant genera of fungi on damp materials in residential housing are species of *Aspergillus* and *Penicillium* (Flannigan & Miller 2001). Their presence has been related to indicators of dampness (Dales et al. 1997), and, in some studies poor respiratory health (Gent et al. 2002). Most of the 58 species of *Penicillium* subgenus *Penicillium* produce toxins (Samson and Frisvad 2004), including the species encountered in the indoor environment.

In this study, we evaluate the dose response and time course profiles associated with inflammatory and cytotoxic responses in mouse lungs intratracheally instilled with pure

brevianamide A and mycophenolic acid, the dominant metabolites of *P. brevicompactum* Dierckx and roquefortine C, common in strains of *P. chrysogenum* Thom, respectively (Samson and Frisvad 2004). These species are commonly encountered in wood framed buildings with moisture problems. Previous *in vivo* studies indicated that *Penicillium* spores and spores extracts dose-dependently stimulate inflammatory and cytotoxic lung responses (Schwab et al 2004; Chung et al. 2005). We hypothesized that pure toxin exposures would elicit such responses in a mouse model of lung disease. In addition, we hypothesized that the pharmacokinetics and effects on lung biology would be different.

## **Materials and Methods**

### ***Toxins***

The compounds used in these studies were isolated during studies of the toxigenic potential of building-associated fungi. Brevianamide A was isolated as a yellow solid; melting point 200-212°C (lit. 190-220°C). Mycophenolic acid was isolated as white crystals; melting point 141-142°C (lit. 139-141°C). Roquefortine C was obtained as white crystals, melting point 195-200°C (lit. 195-200°C). For all three compounds, carbon and proton NMR spectra, optical rotations and the IR maxima were similar to the literature values. Purity based on H NMR, melting point and other spectral analysis was >98% (Birch and Wright 1970; Cole and Schweikert 2003, Puniani et al. 2005). The structures of all three compounds are shown in Figures 1a-c.

### ***Animal Studies***

For the animal studies, brevianamide A and mycophenolic acid were dissolved in 10 % ethanol in physiological saline. They were then diluted to working solutions and administered intratracheally as a single dose (see below) at concentrations of either 0.5 nM, 2.5 nM, 5.0 nM or

12.5 nM/g body weight (BW) animal. Roquefortine C, which is less water soluble than the other two toxins (see Bünger et al. 2004), was first dissolved in 0.02 % Tween then added to 10% EtOH in physiological saline, then diluted to working solutions and administered intratracheally as a single dose at concentrations of either 0.5 nM, 2.5 nM, 5.0 nM or 12.5 nM/g BW animal. Toxin concentrations were selected based on some of our earlier studies on *Stachybotrys chartarum* toxins and spores (Mason et al., 1998; Hastings et al. 2005; Rand et al. 2005) where it was found that they cause significant and reproducible rodent lung inflammation onset without severe morbidity.

### ***Animals***

Random-bred pathogen-free Swiss Webster Carworth Farms white (CFW) 21-28 day old male mice (23.2±2 g BW) were used in this experiment. The mice were housed according to the standards of the Canadian Council for Animal Care (CCAC, 1993) and with approval from the Dalhousie University Animal Care Committee. The mice were given food and water ad libitum and acclimatized for one-week prior to use.

### ***Experimental design***

A total of 300 mice were divided into 4 treatment groups (control, brevianamide A, mycophenolic acid or roquefortine C). The treatment animals (either brevianamide A, mycophenolic acid or roquefortine C) were separated into 4 groups of 20 mice each. To test for dose dependent effects, treatment mice were exposed to a single dose of each toxin (see above) by intratracheal instillation. At each dose used, time course of effects was studied at 3h, 6h, 24h and 48h post instillation (PI). Two groups of 20 control animals were used. One group was intratracheally instilled with 10 % EtOH in physiological saline carrier, the other was instilled with 0.02%Tween/10% EtOH in physiological saline.

### ***Intratracheal instillations and lung recovery***

Before instillation, mice were lightly anaesthetized with an intramuscular injection of a ketamine (Ketaleen) and xylazine (Rompun) mixture. Once anaesthetized, each mouse was weighed then placed on an intratracheal instillation board 20° from the vertical as described in Mason *et al.* (1998) and instilled with 50µl of toxin or EtOH saline carrier. Mice were left in the upright position on the instillation board for approximately two minutes before being placed back in their cages on a warm pad to recover. During recovery, mice were monitored for signs of sickness or distress as outlined in CCAC guidelines (CCAC, 1993).

Bronchioalveolar lavage fluid (BALF) recovery.

Mice in each of the three treatment groups were sacrificed 5 at a time at 3, 6, 24 or 48hr post instillation (PI) with a 300µl intraperitoneal injection of 65mg/ml of sodium pentobarbital (Somnotol). They were exsanguinated by cutting the abdominal artery. Mouse lungs were then lavaged with 0.9% physiological saline in 4 x 0.8 ml aliquots and pooled as previously described by Mason *et al.* (1998). Protease inhibitors, PMSF at a final concentration of 0.1 mM and EDTA at final concentration of 5 mM were added to the pooled BALF. BALF from each mouse was centrifuged at 300 g for 5 min at 4° C. The cell pellet was used for cell enumeration and differentiation while the supernatant was used for pro-inflammatory chemokine/cytokine and lactate dehydrogenase activity analysis (see below).

*BALF cell counts.*

The cell pellet was resuspended in 1 ml ice-cold physiological saline solution and an aliquot removed for cell counting using a hemocytometer. A slide was prepared from a 100 µl aliquot of the suspension using a Cyto-Tek® (Miles Scientific) centrifuged at 1500 rpm for 5



min. The cells were then stained with Diff-Quik® solution (Fisher Scientific) and differentiated at 200 nucleated cells per slide.

#### ***Chemokine and pro-inflammatory cytokine analysis.***

Concentrations of the chemokine MIP-2 and the pro-inflammatory and oxidative stress cytokines IL-6 and TNF- $\alpha$  were determined using sandwich ELISA procedures that were purchased from BD Biosciences. Assay diluent and tetramethyl benzoate (TMB) substrate reagent sets were also purchased through BD Biosciences. All standard curves for ELISA kits were generated according to the respective kit instructions. Final absorbencies were read at 450nm using a Bio-Tek Instruments EL-800 microplate reader. TNF- $\alpha$  and MIP-2 concentrations were reported in pg/ml of BALF.

#### ***Lactate dehydrogenase(LDH) activity analysis.***

LDH activity levels were measured as an indicator of cytotoxicity. Concentrations were determined using the enzyme based Cytotoxicity Detection Kit (Roches Diagnostics) as previously described (Flemming et al., 2004). Briefly, a standard curve was generated for the quantification of LDH levels by serially diluting an L-LDH solution (Roche Diagnostics) in physiological saline (0.9% NaCl) to concentrations of 10, 25, 75, 100, 125 and 150 ng/ml. The final absorbance of the solutions was determined at the 490 nm wavelength using the microplate reader. LDH activity levels were reported as ng/ml of BALF.

#### ***Albumin***

Albumin concentrations in BALF, an indicator of vascular permeability, were quantified using a quantitative enzyme linked immunosorbent assay (ELISA) purchased from Bethyl Laboratories Inc. Samples were diluted 1:800 in diluent (Tris-buffered saline (TBS) with 1% bovine serum albumin (BSA), 0.05% Tween 20, pH 8.0). Final absorbencies were determined

using a Bio-Tech Instruments® ELx800 automated microplate reader at  $\lambda$  450 nm. Albumin concentrations were reported in ug/ml of BALF.

### ***Statistical Analysis***

All data were tested for normality using a normal probability plot and were transformed to improve normality and homoscedasticity using a log transformation. Data were then tested for statistical significance using one-way ANOVA. Tukey's multiple comparison test was used to examine differences between control- and treatment animal data. All data are shown as mean  $\pm$  SE. Statistical analysis was carried out using SYSTAT version 8.0 and results were considered significant at  $\leq 0.05$  probability level.

### **Results**

None of the control, brevianamide A or mycophenolic acid treatment animals used in the experiments showed signs of illness or respiratory distress. However, high dose roquefortine C animals showed signs of trembling and lethargy until 24 post instillation (PI). Furthermore, lungs of these animals were paler than those of the other treatment animals (data not shown). Compared to controls, none of animals treated with 0.5 nM (data not shown) or 2.5 nM/g toxin body weight (BW) showed significant responses in any of the parameters evaluated.

#### ***Average animal weight change.***

Average weight change was similar for all animal groups. For all treatment groups, average weight change was slightly depressed for up to 6 hr PI then increased modestly until 48 h PI. However, there were no statistically significant dose or time dependent differences in average weight change amongst animal groups ( $p > 0.05$ ) (data not shown).

#### ***Bronchioalveolar lavage fluid (BALF) cell counts.***

BALF macrophage and neutrophil numbers in control and treatment mice are shown in Fig. 2 a – f. Brevianamide A, mycophenolic acid and roquefortine C differentially induced significant production of these two cell types. Compared to control levels, macrophage and neutrophil numbers in animals treated with 12.5 nM/g toxin BW were increased to significant levels starting at 6 h PI, and at 24 h PI in animals treated with  $\geq 5.0$  nM/g BW brevianamide A or roquefortine C. Additionally, both macrophage and neutrophil numbers showed dose-dependent like responses in high dose brevianamide A and roquefortine C treatment animals at either 24 and/or 48 h PI (Fig. 2 a-f). Neutrophil numbers also exhibited a dose-dependent like response in mycophenolic acid treatment animals.

Eosinophil or lymphocyte numbers in treatment groups were not significantly different from those of control animals (data not shown).

#### ***Pro-inflammatory chemokine/cytokines.***

Brevianamide A, mycophenolic acid and roquefortine C differentially induced significant tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), macrophage inflammatory protein-2 (MIP-2) and interleukin-6 (IL-6) production (Figs. 3 - 5). In mice exposed to  $\geq 5.0$  nM/ g BW brevianimide A, TNF- $\alpha$  levels showed a significant increase from 6 to 48 hr PI (Fig. 3a). Compared to control levels, MIP-2 was significantly elevated in mice exposed to 12.5 nM/g BW brevianimide A from 3 to 48 hr PI. Additionally, they were dose-dependently increased in the 5.0 and 12.5 nM/g BW brevianimide A treatment groups at 48 h PI (Fig. 3 b). IL-6 concentration was significantly elevated only in animals exposed to 12.5 nM/g BW brevianimide A and at 6 h PI. (Fig. 3 c).

Mice treated with mycophenolic acid showed relatively few significant pro-inflammatory responses and only in the 12.5 nM/g BW treatment group. For TNF- $\alpha$ , concentrations were significantly increased at 6 to 48 h PI; for MIP-2 they were significantly elevated from 24 to 48 h

PI (Figs. 4 a, b). Compared to controls, IL-6 levels were not significantly changed in any of the mycophenolic acid treatment groups (Fig. 4 c).

In mice intratracheally exposed to roquefortine C, TNF- $\alpha$  levels were significantly elevated from 6 to 48 h PI in animals receiving 12.5 nM/g BW and at 6 to 24 h PI in the 5.0 nM/g BW treatment group (Fig. 5a). In  $\geq 5.0$  nM/g BW roquefortine C treatments, MIP-2 levels were significantly elevated only from 6 to 24 h PI before returning to control levels (Fig. 5 b). IL-6 levels were not significantly changed in any of the roquefortine C treatment groups compared to controls (Fig. 5 c).

### ***Cytotoxicity.***

LDH activity levels in BALF were measured as a non-specific marker of cellular damage. Significantly increased LDH activity levels were observed only in the BALF of mice treated with high dose (12.5 nM/g BW) brevianamide A and at 6 and 24 h PI (Fig. 3 d).

### ***Albumin.***

Albumin concentration was measured as a non-specific marker of vascular leakage. Significant albumin levels were detected in the BALF of mice treated with 12.5 nM/g BW brevianamide A (Fig.3 e) and with  $\geq 5.0$  nM/g BW mycophenolic acid (Fig 4 e) both at 6 to 24 h PI. Albumin concentrations were statistically unchanged from control in all roquefortine treated animals (Fig. 5 e).

## **Discussion**

It is clear from *in vitro* and *in vivo* studies that *Penicillium* spores and spore extracts can induce significant immunomodulatory responses in lung cells, and inflammation in animal models of lung disease (Chung et al. 2005; B nger et al. 2004; Huttunen et al. 2004; Schwab et al. 2003, 2004; Jussila, et al. 2002; Cooley et al. 1999, 2000; Licorish et al. 1985). However,

because spores and spore extracts comprise mixtures of bioactive constituents often including toxins, it is impossible to resolve which constituent mediates inflammatory responses. Jussila et al. (2002) suggested that  $\beta$  glucans sequestered in walls of this species might be responsible.  $\beta$  glucans are known to be potently pro-inflammatory (Brown and Gordon 2001; Vassallo et al. 2003) and exhibit acute pulmonary toxicity (Fogelmark et al. 1992; Young et al. 2003). Moreover, other studies have shown that allergens in *Penicillium* spore and hyphal walls can also provoke inflammatory responses (Chung et al. 2005; Schwab et al. 2003, 2004; Cooley et al. 1999, 2000). However, Bunger et al. (2004) have suggested that adverse health effects may also be exacerbated or caused by exposure to *Penicillium* toxins.

Amongst the *Penicilli*, the predominant taxa encountered in mold contaminated building environments are in *Penicillium* subgenus *Penicillium*. All of these species can produce low molecular weight toxins (Samson and Frisvad 2004) although for most, little is known about their relative potency. A few have been tested for cytotoxicity to mammalian cell lines and primary cultures of rodent alveolar macrophages (Sorenson 1986; Sorenson et al. 1986; Bunger et al. 2004), virtually nothing is known about how they impact on lung health *in vivo*.

In this study, brevianamide A and mycophenolic acid from *P. brevicompactum* and roquefortine C from *P. chrysogenum* induced significant inflammatory responses expressed as differentially elevated MIP-2, TNF, and IL-6 concentrations in the bronchioalveolar lavage fluid (BALF) of intratracheally exposed mice. It was also found that the highest dose brevianamide A induced cytotoxicity manifested as significantly increased LDH concentration in mouse BALF. Brevianamide A and mycophenolic acid exposures also stimulated vascular leakage, observed as increased BALF albumin concentrations in lungs of high dose treatment animals. While these results provide evidence that *Penicillium* spp. toxins can induce inflammatory lung responses, it

remains unclear how the toxin concentrations used in this study compare to spore loads. Unlike for *Stachybotrys chartarum* where toxin equivalency for spores has been documented (Chung et al. 2003; Yike et al. 1999), toxin concentration in *Penicillium* spp. spores is unknown. However, the concentrations selected for this study were based on some of our earlier studies on *S. chartarum* that were designed to represent toxicological potency associated with low to moderate (= 30 to 3000 spores/g BW) spore exposures.

It was hypothesised that inflammatory responses induced by exposure to the three *Penicillium* toxins would exhibit dose-dependent like patterns. Such responses have been documented in a variety of *in vitro* and *in vivo* studies of exposures to non-biological particulates, bacteria and bacterial endotoxin, and fungal spores, allergens and toxins (Chung et al. 2005; Flemming et al. 2004; Jakab et al. 1994; Mason et al. 1998; Mi-Gyung et al. 1999; Rand et al. 2005). However, dose dependent responses were observed for only macrophage (all three toxins) and neutrophil (brevianamide A and roquefortine C treatments) numbers and MIP-2 (brevianamide A treatment). Possibly, lack of clear dose-dependent response patterns in the other inflammatory and cytotoxic parameters tested may be due to the narrow toxin concentration range used, relative lack of sensitivity of the non-cellular parameters tested, toward *Penicillium* spp. toxins, especially IL-6, or to the fact that exposure was through intratracheal instead of inhalation route. Chung et al. (2005) recently revealed that *P. chrysogenum* allergens triggered significant and dose dependent changes in lung cell numbers, serum and BALF IgE levels and BALF IL-5, amongst others, in mice exposed through inhalation. Nevertheless, while dose-dependent like responses were not induced in the cytokine, albumin and cytotoxic parameters tested, significant changes were noted, and mostly in 12.5 nM/g BW toxin treatment animals. These results were interesting because the *Penicillium* toxins used in this study were not

previously known to potentiate inflammatory responses in mammalian cells. Brevianamide A exhibits insecticidal properties (Gloer 1995), mycophenolic acid is used to suppress immune grafting reaction in organ transplant patients (Bentley 2000), and roquefortine C is neurotoxic (Arnold et al., 1987).

The observation that roquefortine C can stimulate significant inflammatory responses *in vivo* might help explain some of the indoor effects associated with *P. chrysogenum* spore exposures in indoor environments. *Penicillium chrysogenum* is the most widely encountered *Penicillium* species in the built environment. The different biotypes of this species in indoor environments are important food contaminants, and are the predominant mycospora in building dust and on a variety of water damaged building surfaces (Scott et al. 2004), including wallboard, solid wood surfaces and ceiling tiles, amongst others (Flannigan & Miller 2001). This species had not been considered to produce mycotoxins i.e. compounds known to affect human or animal health from ingestion exposure (Nielsen and Gravesen 1999). However, with the new understanding that building-associated strains represent a different population from those encountered outdoors, this view may need to be revised. Building-associated strains of *P. chrysogenum* produce roquefortine C (Samson and Frivad 2004), including all Canadian building isolates tested (Miller et al. unpublished data).

The responses induced by fungal spore and toxin exposures appear to generally reflect both general pulmonary and cell-specific reactions (Flemming et al. 2004; Rand et al. 2005). Amongst the general pulmonary reactions observed in this study were significantly increased albumin and inflammatory cells (macrophages and neutrophils) concentrations in lungs of high dose treatment animals. Albumin production is a reflection of increased vascular leakage and a consequence of molecular, biochemical and cellular events resulting from the production and

release of endogenously derived substances such as TNF $\alpha$ , IL-6 and endothelins (Guimaraes et al. 2002; Thickett et al. 2001). Additionally, it can also be a consequence of more generalized responses such as increased blood perfusion pressure, and vascular deficit (Flemming et al. 2004). Inflammatory cell influx into the bronchioalveolar space (BAS) of mice after toxin instillation also appears to be a non-specific response of the lower respiratory tract because it is induced by a variety of stimuli including LPS, viable organisms, inhaled particulates, toxic gases, and fungal extracts and metabolites, amongst others (Cooley et al., 1999, 2000, Henderson 1984, Henderson et al., 1985, Huttunen et al., 2004; Lopez, 1986; Nielson et al., 2002; Rand et al. 2005; Yike et al., 2005). It is also controlled by host-derived cytokines, which in turn mediate lung inflammation by serving as leukocyte chemoattractants and leukocyte-activating, amongst others (Standiford et al., 1999). Interestingly, when we compared the ratios of magnitude of cellular change (cell treatment / control cell numbers) to the magnitude of cytokine/chemokine responses (treatment immunomodulator/control values) in treatment animals the calculated ratios were well within the range of those of other pneumonotoxins (Lopez, 1986), which suggests that *Penicillium* toxin treatments did not induce a disproportionate inflammatory response characterized by exaggerated cellular influx. As these general responses are induced by a variety of exposures, they are not toxin-specific. On the other hand, the pattern of cell-specific responses, especially those related to cytokine expression, may provide insight useful for the development of biomarkers of mold toxins.

A result common to studies of the effects of mold spores and toxins is differential expression of MIP-2, a member of the C-X-C family of cytokines, and/or TNF $\alpha$  by lung cells *in vitro* and *in vivo* (see Shahan et al. 1998; Liew et al. 2003; Hudson et al. 2004; Rand et al. 2005). Both MIP-2 and TNF- $\alpha$  are involved in acute pulmonary inflammation and induced by a



wide range of cells including alveolar macrophages (AM's), mast cells, epithelial cells and other lung-associated cells (Driscoll et al., 1995). In a series of *in vitro* studies, Shahan et al. (1998) found that latex spheres stimulated little or no C-X-C chemokine and TNF- $\alpha$  up-regulation in rat BALF cells compared to fungal spore exposures suggesting that fungal spores not latex spheres are immunomodulatory. They also found that the magnitude and temporal patterns of MIP-2, KC, TNF- $\alpha$ , MIP-1 and  $\beta$ -actin mRNA expression in the rat cells exposed to spores of *Aspergillus fumigatus*, *A. niger*, *A. terreus*, *P. spinulosum* and *Cladosporium cladosporioides* spores varied markedly. Rapid mRNA up-regulation and expression of the CXC chemokines was detected in the BALF cells exposed to *A. fumigatus*, *A. niger* and *C. cladosporioides*, but not in those exposed to *A. terreus* or *P. spinulosum* spores. Moreover, they also found that tyrosine phosphorylation of specific proteins associated with C-X-C chemokine expression differs with the fungal species, especially those capable of stimulating differential C-X-C chemokine up-regulation. Results of these *in vitro* studies are supported by the *in vivo* studies of Leino et al. (2003) and Hudson et al. (2005), which showed that MIP-2 mRNA expression is differentially up-regulated in mouse lungs exposed to trichothecene producing and non-producing *Stachybotrys chartarum* spores. Rand et al. (2005) also found differences in MIP-2 concentrations in mouse BALF exposed to atranones A and C from *S. chartarum* suggesting that differences in toxin potency may influence expression of this chemokine. These results although based on a few fungi and toxins, are compelling because they all suggest that this chemokine family responds differentially to fungal spores and toxins. Why expression of the C-X-C chemokine family is so sensitive to fungal spore and toxin exposures is unclear and deserves further attention.

In mice, intratracheal exposure to brevianamide A, mycophenolic acid and roquefortine C provoked compound-specific toxic responses with different toxicokinetics. This result is important for several reasons. The data provide support for previous studies suggesting that fungal spores, their extracts and toxins result in differing inflammatory and/or cytotoxic response patterns. There are evidently many factors that can affect fungal spore- and/or toxin-induced lung inflammation: particle size of the fungal materials in air, species specific variations in glucan concentration (Foto et al. 2004), toxins present and their concentration, and as we have shown, in toxicokinetics of inflammatory mediator release, toxin targeting, and different toxin clearance rates, amongst others. Variations in any these factors would affect the dynamics and outcome of lung inflammation at the whole animal level. Lastly, the results are important because they support other studies (Flemming et al. 2004; Hudson et al. 2005; Leino et al. 2003; Rand et al. 2005; Shahan et al. 1998) that provide results indicating that differences in the lung inflammatory response patterns stimulated by fungal spore and toxin exposures might prove to be important in development of biomarkers of exposure.

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### Figure captions

Figure 1a. Structure of brevianamide A from *P. brevicompactum*; 1b. structure of mycophenolic acid from *P. brevicompactum*; 1c. Structure of roquefortine C from *P. chrysogenum*.

Figure 2 (a – f). Dose and time profiles for macrophage and neutrophil numbers in BALF of mice exposed to brevianamide A (a, b) mycophenolic acid (c, d) and roquefortine C (e, f). Data are mean  $\pm$  S.E.M. a = 12.5 nM/g BW dose/animal significantly different than all other treatments; b = 5.0 and 12.5 nM/g BW dose/animal similar and significantly different than controls; c = 12.5 nM/g BW dose/animal > 5.0 nM/g BW dose/animal > than control.

Figure 3 (a – e). Dose and time profiles for TNF $\alpha$ , MIP-2, IL-6, LDH activity and albumin in BALF from brevianamide A treated animals. Data are mean  $\pm$  S.E.M. a = 12.5 nM/g BW dose/animal significantly different than all other treatments; b = 5.0 and 12.5 nM/g BW dose/animal similar and significantly different than controls; c = 12.5 nM/g BW dose/animal > 5.0 nM/g BW dose/animal > than control.

Figure 4 (a – e). Dose and time profiles for TNF $\alpha$ , MIP-2, IL-6, LDH activity and albumin in BALF from mycophenolic acid treated animals. Data are mean  $\pm$  S.E.M. a = 12.5 nM/g BW dose/animal significantly different than all other treatments; b = 5.0 and 12.5 nM/g BW dose/animal similar and significantly different than controls.

Figure 5 (a – e). Dose and time profiles for TNF $\alpha$ , MIP-2, IL-6, LDH activity and albumin in BALF from roquefortine C treated mice. Data are mean  $\pm$  S.E.M. a = 12.5 nM/g BW dose/animal significantly different than all other treatments; b = 5.0 and 12.5 nM/g BW dose/animal similar and significantly different than controls.

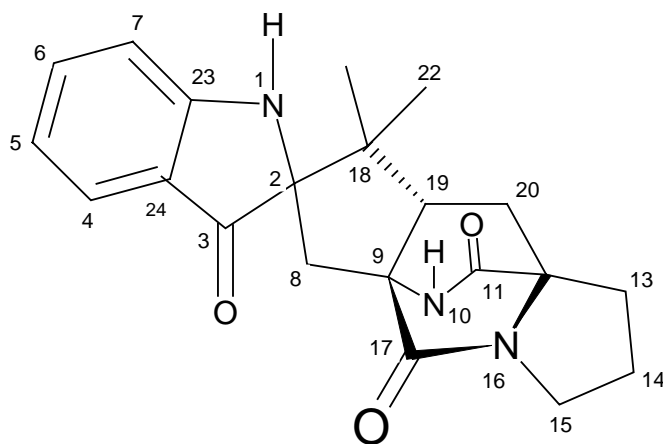


Figure 1a.

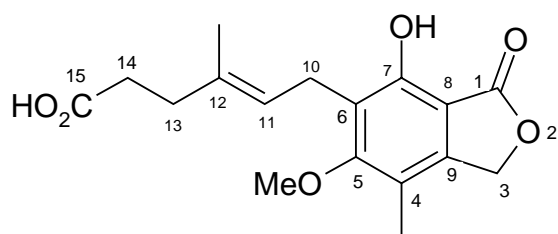


Figure b.

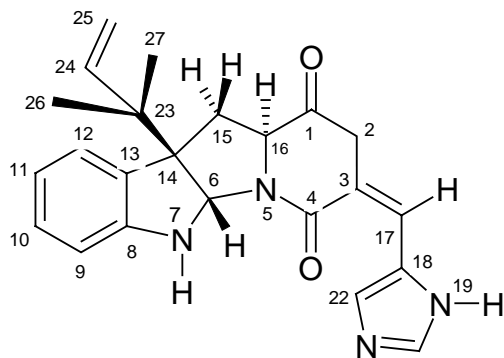


Figure c.









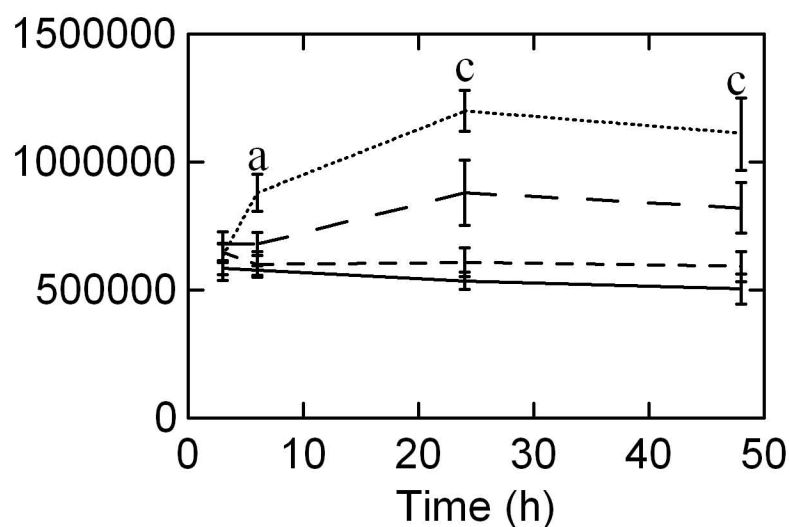


# TREATMENT

- Control
- - - 2.5 nM/g
- - - 5.0 nM/g
- ..... 12.5 nM/g

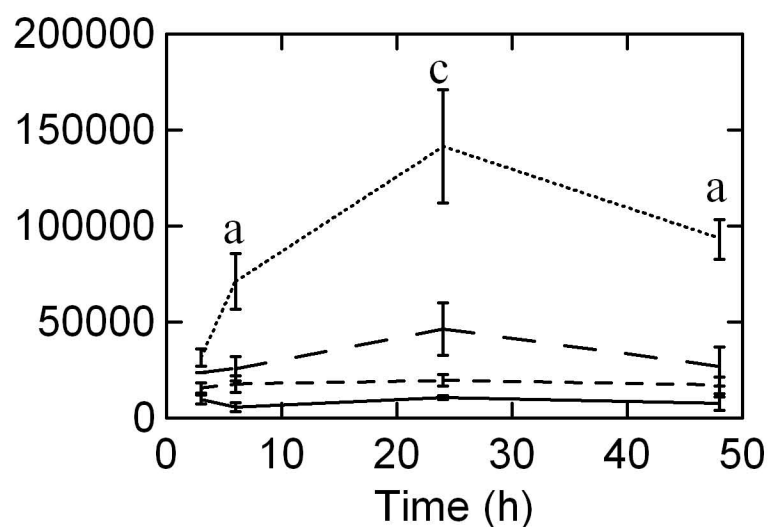
Macrophage number/ml BALF

**a**

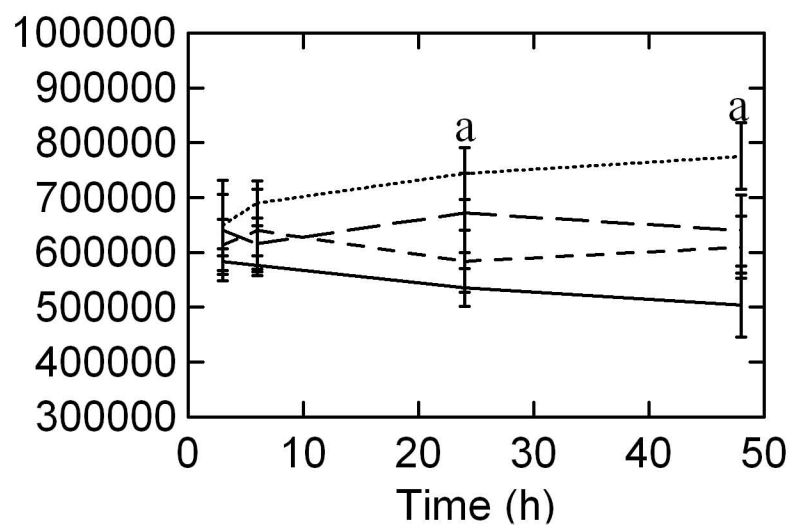


Neutrophil number/ml BALF

**b**

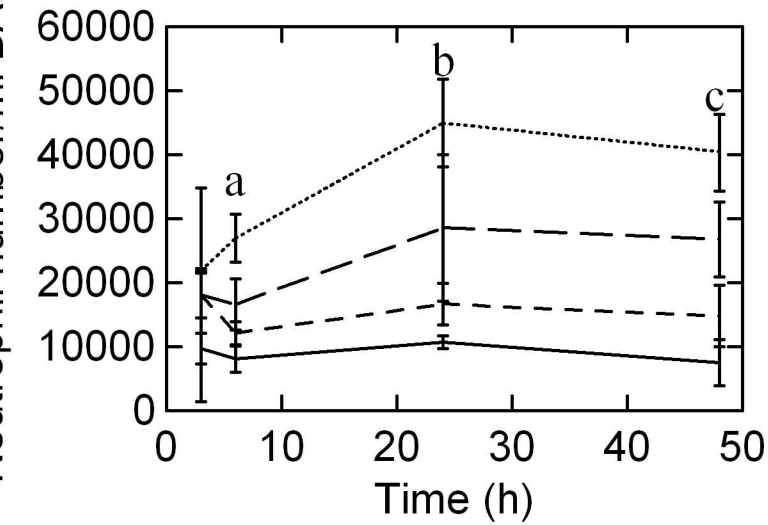


**c**

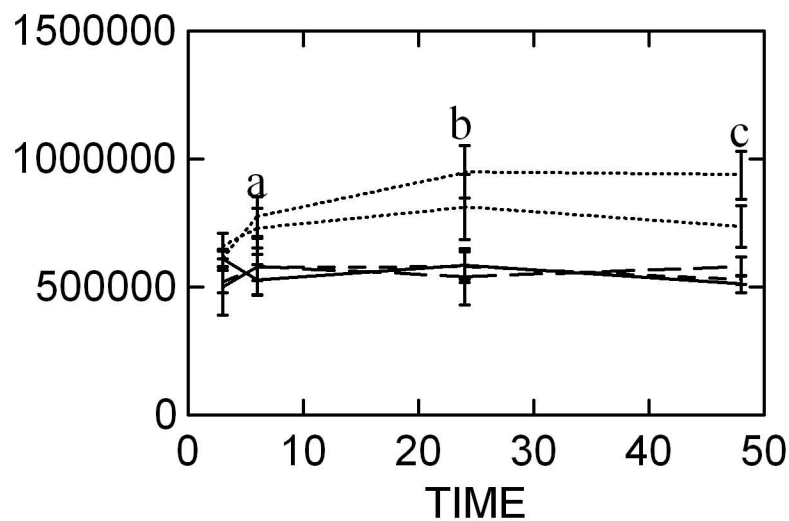


Neutrophil number/ml BALF

**d**

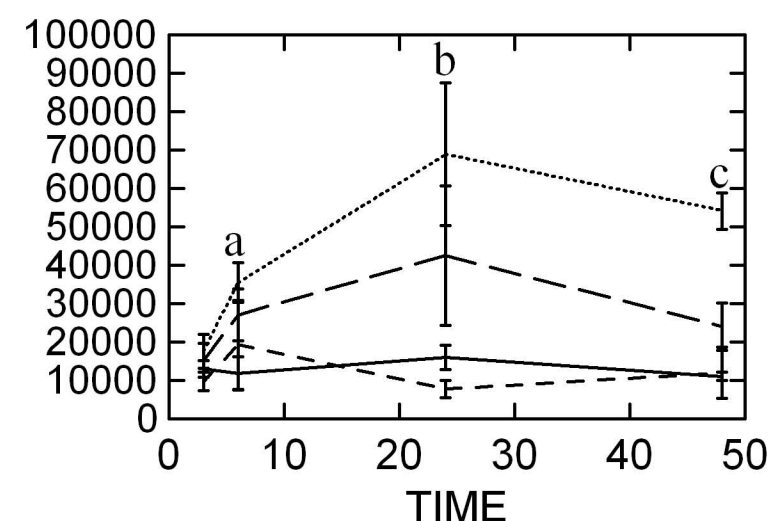


**e**



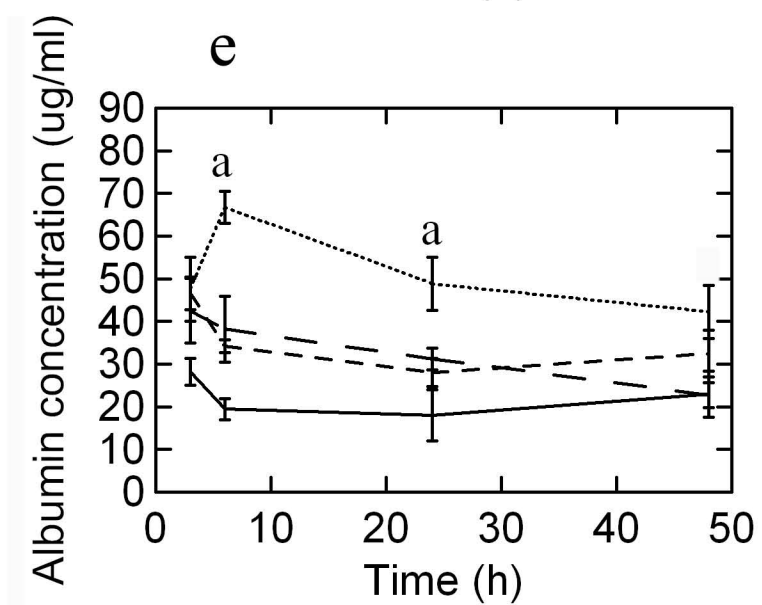
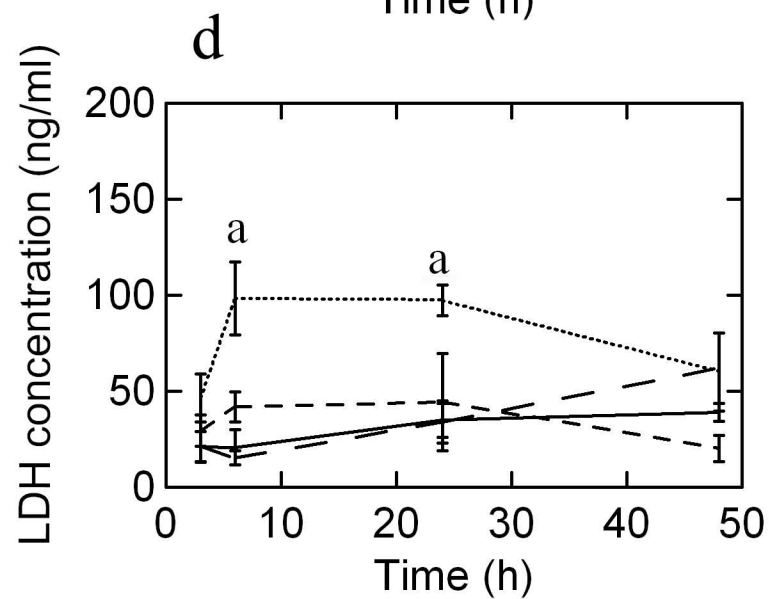
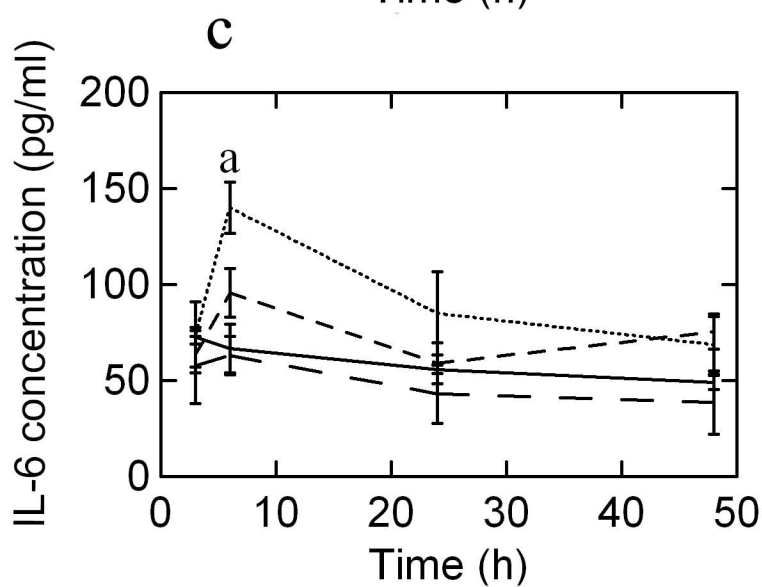
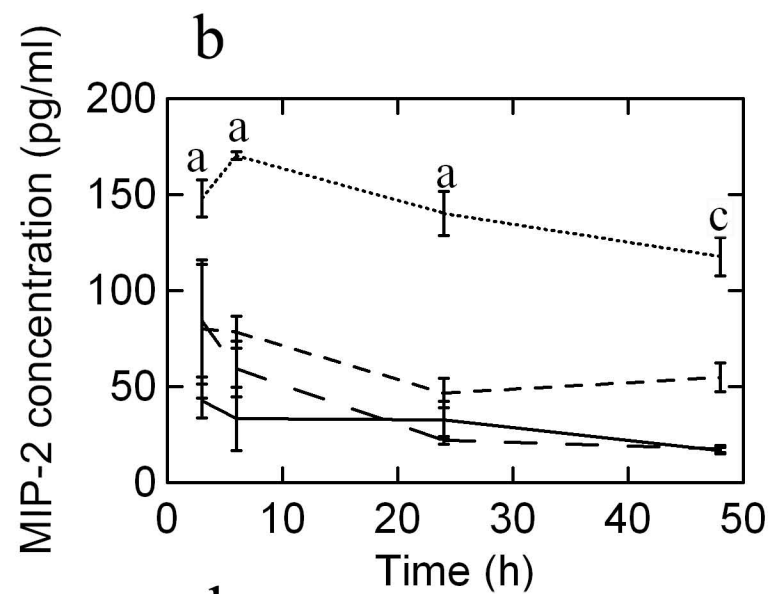
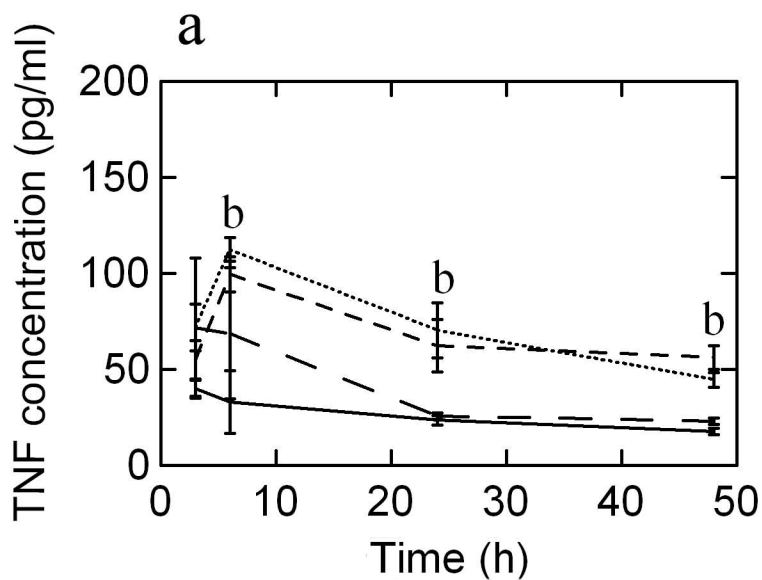
Neutrophil number/ml BALF

**f**



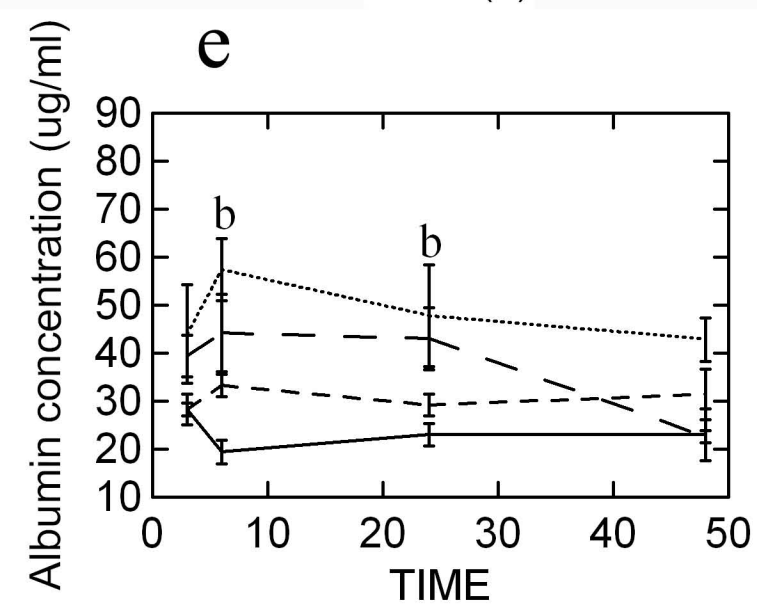
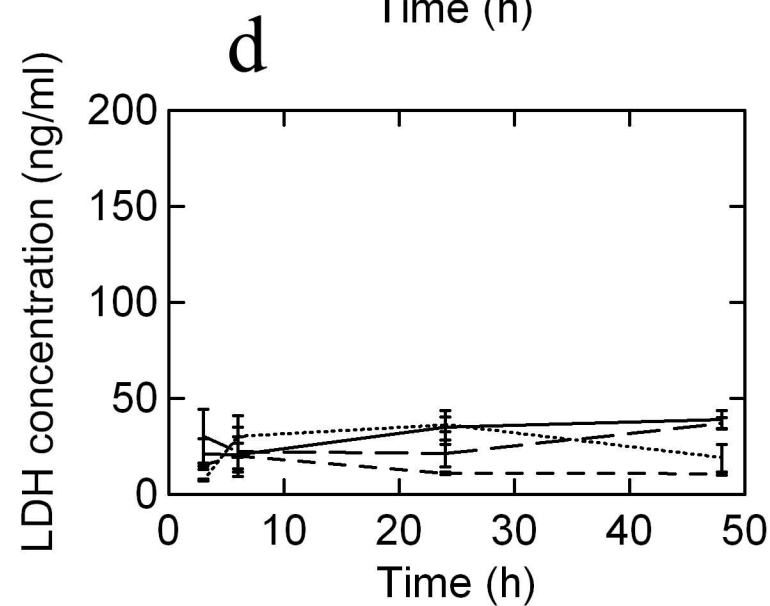
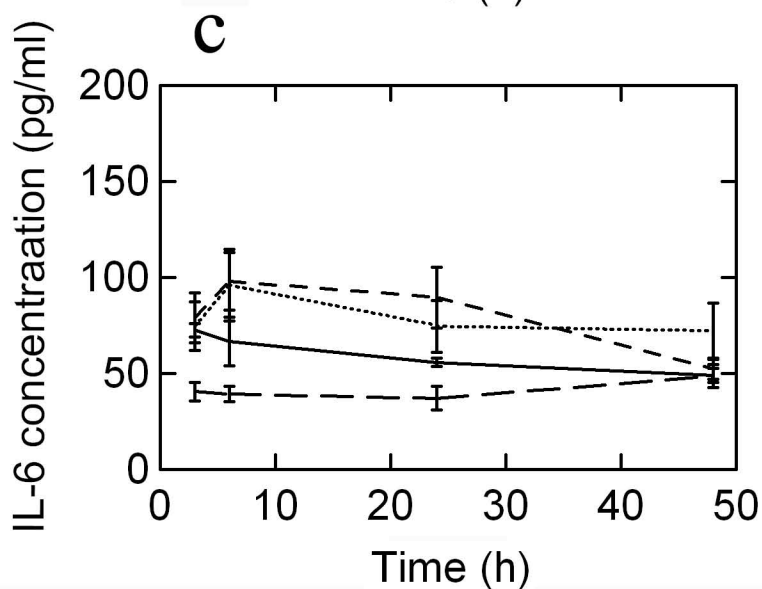
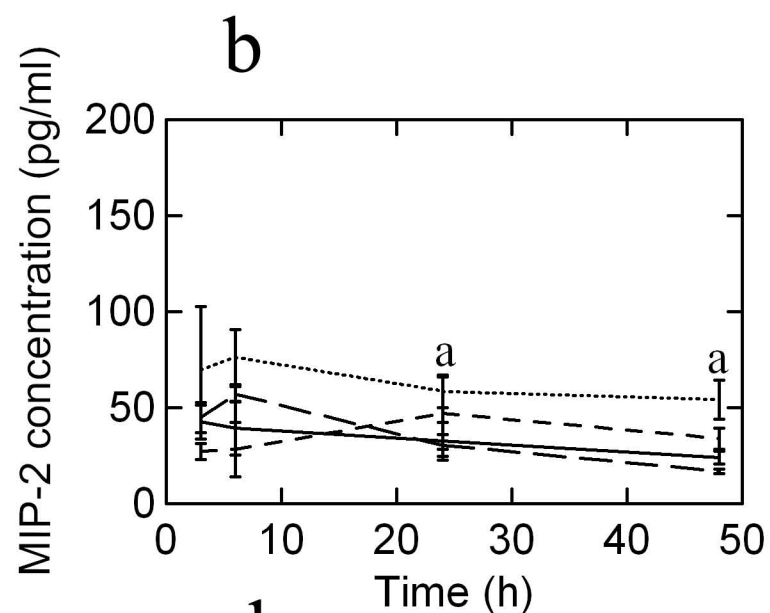
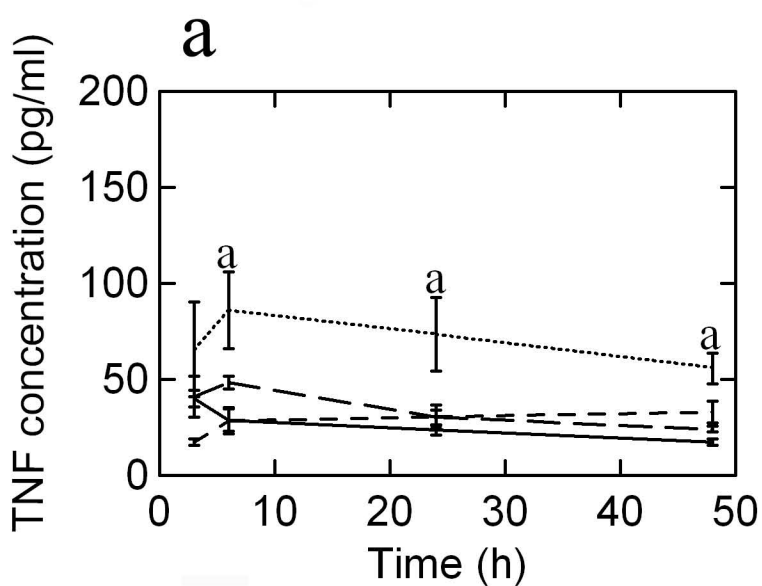
# TREATMENT

- Control
- - - 2.5 nM/g
- . - 5.0 nM/g
- ..... 12.5 nM/g



# TREATMENT

- Control
- - - 2.5 nM/g
- - - 5.0 nM/g
- ..... 12.5 nM/g



# TREATMENT

- Control
- - - 2.5 nM/g
- - - 5.0 nM/g
- ..... 12.5 nM/g

